Biochimica et Biophysica Acta xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Review

Biophysics and function of phosphatidic acid: A molecular perspective

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ARTICLE INFO

Article history:
Received 9 January 2009
Received in revised form 29 March 2009
Accepted 1 April 2009
Available online xxxx

Keywords:
Electrostatic-hydrogen bond switch
Lipid–protein interaction
Model membrane
MAS NMR
Phosphomonoester
Anionic lipid
Hydrogen bond

ABSTRACT

Phosphatidic acid is the simplest (diacyl)glycerophospholipid present in cells and is now a well established second messenger with direct biological functions. It is specifically recognized by diverse proteins and plays an important role in cellular signaling and membrane dynamics in all eukaryotes. An important determinant of the biological functions of phosphatidic acid is its anionic headgroup. In this review we will focus on the peculiar ionization properties of phosphatidic acid and their crucial role in lipid–protein interactions. We will take a molecular approach focusing entirely on the physical chemistry of the lipid and develop a model explaining the ionization properties of phosphatidic acid, termed the electrostatic-hydrogen bond switch model. Diverse examples from recent literature in support of this model will be presented and the broader implications of our findings will be discussed.

1. Introduction

Phosphatidic acid (PA) is the simplest diacyl-glycerophospholipid and occurs only in small amounts (often less than a few mol%) in biological membranes but yet is crucial for cell survival. This is related to its central role in glycerophospholipid synthesis [1], and also to its diverse functions in lipid signaling and membrane dynamics [2–5]. The chemical structure of PA consists of alcohol, glycerol, to which two fatty acids (also named acyl-chains) and a phosphate are esterified at positions 1, 2 and 3, respectively (see Fig. 1). The anionic phosphate headgroup is attached as a phosphomonoester and it is this defining feature that sets PA apart from all other diacyl-glycerophospholipids. Therefore, the specificity of PA–protein interaction is likely related to the ionization properties of this phosphomonoester headgroup. In this review we will focus on the electrostatics of PA, and more specifically, on the factors that regulate the negative charge of the phosphomonoester headgroup. The ionization properties of PA and their role in the PA–protein interaction will be summarized in the electrostatic-hydrogen bond switch model.

High affinity phosphatidic acid–protein interaction has been observed for over 22 proteins in mammalian, plant and yeast cells. Most of these have been reviewed in two excellent papers by Stace and Testerink [6,7] . Interestingly, when one compares the PA binding region of the subset of proteins in which the binding region has been evaluated, no consensus sequence (sequence homology) is apparent, in sharp contrast to other lipid binding modules such as PH, PX, C1 and C2 domains. One general, but not surprising, feature that arises is the presence of basic amino acid residues. In most cases hydrophobic residues appear to be important as well. In order to understand how PA-binding proteins recognize PA among other anionic glycerophospholipids and to understand how PA may function in general in biological membranes, we set out to determine the ionization properties of the phosphomonoester headgroup of PA. First, we will briefly review the role of anionic lipids in biological membranes, and discuss what factors influence ionizable groups. This technical section also briefly discusses how the negative charge of PA (and other phospholipids) is determined and can be skipped upon a first reading of this review. Subsequently, we will discuss how the ionization properties of PA can be determined, review the peculiar electrostatics of PA and discuss biological implications.

2. Protein function and organization are affected by the anionic nature of biological membranes

Biological membranes contain a host of anionic lipids and are therefore generally negatively charged. A good example is the inner leaflet of the plasma membrane which contains well over 30 mol% of anionic lipids [8,9], mainly phosphatidylserine (PS) and phosphatidylinositol (PI) in mammalian cells [10,11]. The negative charge of biological membranes is an important determinant of biomembrane structure and function. It is well established that the negative charge carried by anionic lipids in biomembranes forms an important site of attraction for positively charged (carrying basic amino acids) protein

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1388-1981/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.bbalip.2009.04.001

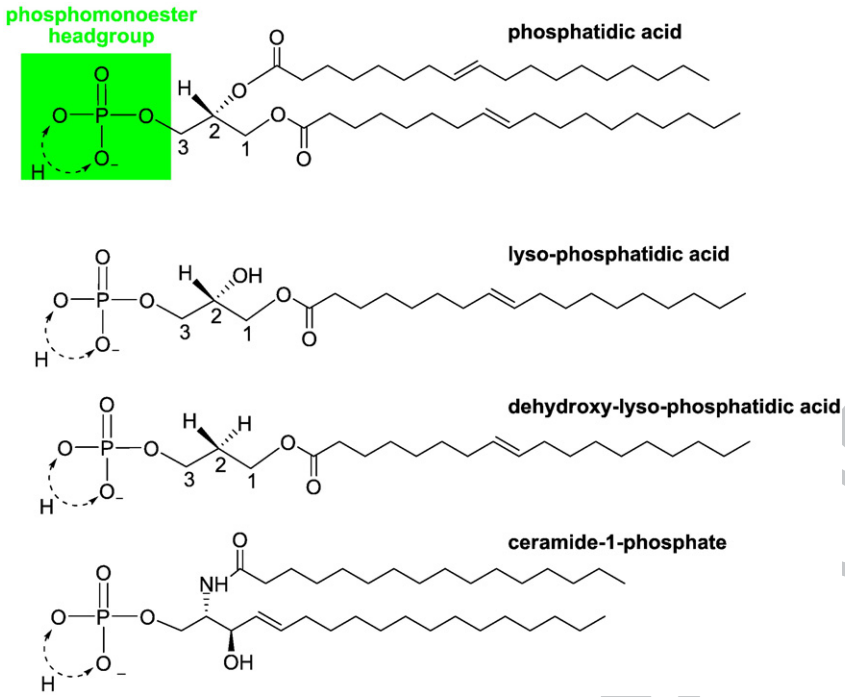

Fig. 1. Chemical structure of phosphatidic acid and several other lipid phosphomonoesters.

domains [12–14]. One well known example is the cytosolic protein, myristoylated alanine-rich C-kinase substrate (MARCKS) that is targeted to anionic membranes by an unstructured protein domain containing 13 basic amino acid residues [15]. In other cases, cytosolic proteins have developed special binding modules that recognize specific anionic lipids such as the polyphosphoinositides (PIPs) [16] and PS [6]. Interestingly, employing a biosensor that specifically recognizes PS, a recent article by Yeung showed that PS concentration affects peripheral membrane protein localization [11]. Negative charge also affects transmembrane proteins that carry clusters of positively charged residues [17], in that these clusters may guide the membrane insertion and orientation of these proteins. This is also known as the positive-inside rule [18].

3. Lipid charge is affected by bilayer organization and local lipid environment

Given that phosphatidic acid is an anionic phospholipid and that membrane charge has important functional implications, what factors influence the charge of membrane lipids in a bilayer? Ionization behavior of lipids is distinct for a membrane embedded lipid as compared to a free lipid in solution. The reason for this is the interface between the hydrophobic interior and hydrophilic lipid headgroups in a membrane system. The dielectric constant undergoes an abrupt change from about 80 in bulk water to about 1 to 3 for the hydrocarbon region. As a result, a charge (as in PHO_4^-, COO^-, and NH_3^+ groups for example) in the interface produces an electrostatic potential in the aqueous phase that is nearly twice that of the same free (point) charge in solution (the so-called mirror effect [19]). This electrostatic potential affects the potential and charge of nearby (point) charges in the membrane and adjacent aqueous phase.

These electrostatic effects can be approximated surprisingly well, by the classical Gouy–Chapman theory of the diffuse double layer, reviewed in [20]. The diffuse double layer is composed of the charged lipid headgroups (anionic) and adjacent counter ions (cationic) in the aqueous phase that are able to freely diffuse into the bulk solution. A detailed theoretical description is beyond the scope of this review, and the interested reader is referred to the following excellent papers [19–21]. However, some main points relevant to the further understanding of PA ionization properties derived from this theory will be briefly discussed below.

One well known effect is that the surface potential (Ψ) of the membrane is proportional to the surface charge density (σ), and an increase in the negative surface charge, e.g. by an increase in PS concentration, therefore results in an increase in the negative electrostatic potential of the membrane. An increase in the negative surface potential attracts additional protons from the bulk solution lowering the interfacial pH. Thus, a general feature of negatively charged lipid membranes is that the local pH at the lipid headgroup–water interface is lower than in the bulk solution (i.e. the proton concentration is higher). Similarly, the presence of a positive charge, for instance, found in the zwitterionic lipid PC, increases the local pH at the lipid headgroup and decreases the apparent pK_a of the phosphate of the same molecule. Indeed, PS decreases the negative charge of PA, and the pK_a of the phosphate of PC is well below that of the first pK_a of PA [22].

The pK_a is the dissociation/association constant that describes the equilibrium between protonated and deprotonated forms of a chemical group. By definition, there are equal amounts of the protonated and deprotonated group at the pH corresponding to the pK_a. The subscript stands for the apparent pK indicating that the measurement of pK is related to bulk pH and not to the pH at the membrane which cannot easily be determined experimentally.

In addition to the surface charge density, the negative surface potential of a membrane containing acidic lipids is decreased by an increase in the salt concentration (c, ionic strength) of the aqueous phase (Ψ is proportional to $1/$) due to adsorption of counter ions into the diffuse double layer. Interestingly, changes in ionic conditions at constant bulk pH, for example due to Ca^{2+} fluxes, lead to changes in the pH at the membrane surface. Cations like Ca^{2+} will displace protons from the interface into the bulk solution thereby decreasing the proton concentration when compared to the situation prior to the increase in ionic strength [21]. Thus the interfacial pH, and the ionic equilibria that control it, greatly influence the charge of membrane lipids.

At physiological pH values ($5<pH<8$), the charge of most phospholipids will not be affected since their ionization equilibria

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(p*K*s) fall (well) outside this pH range. However, the charge of ionizable groups with p*K* values in the physiological pH range will be affected; important examples are phosphatidic acid, ceramide-1-phosphate and all phosphorylated species of phosphatidylinositol.

Apart from the interfacial pH, the charge of ionizable groups can also be influenced by the formation of hydrogen bonds in two distinct ways. Hydrogen atoms (of –OH and –COOH groups, for example) that participate in a hydrogen bond with a hydrogen bond acceptor will be stabilized against dissociation, i.e. dissociation will occur at a lower proton concentration (higher interfacial pH). In this situation hydrogen bonds increase the p*K* of the ionizable group [23–25]. An interesting and important example is found in the lipid cardiolipin where an intramolecular hydrogen bond network appears to stabilize the second proton against dissociation ([24,26], and R. Epand personal communication). Conversely, hydrogen bonds can also facilitate the dissociation of a proton, as will be shown shortly for phosphatidic acid.

4. Ionization properties of phosphatidic acid

The headgroup of phosphatidic acid is attached as a phosphomonoester (see Fig. 1) and thus has the potential to carry two negative charges, in contrast to the single negative charge on the phosphate of more common anionic phospholipids such as PS and PI. In order to determine the degree of ionization of PA at any given pH, we need to determine the ionization constants that describe the proportions in the membrane. These equilibrium constants are denoted as pK_{a1} and pK_{a2}, respectively, where pK_{a2} falls in the physiological pH range ($5<pH<8$). These constants are not easily determined by conventional experimental methods especially for physiologically relevant model membranes which are essentially flat on the scale of lipid molecular area. Hence, we employed a novel NMR technique.

4.1. How can the ionization properties of the phosphomonoester be probed?

The isotropic chemical shift as determined by ^{31}P NMR of a phosphomonoester is especially sensitive to the degree of dissociation of its two hydroxyl oxygen atoms. This is due to the effect of chemical shielding of the phosphorus nucleus by these oxygen atoms, where deprotonation leads to de-shielding. This effect is largest for the second ionization step which can be monitored very accurately.

Previous ^{31}P NMR work for PA indeed demonstrated the usefulness of this approach [27–29]. However, these studies were limited to unphysiological systems namely, micelles and/or small unilamellar vesicles which give rise to isotropic chemical shifts in solution (static) NMR experiments allowing straightforward analysis. Multi-lamellar vesicle dispersions, with a more physiologically relevant membrane curvature give rise to static spectra that have large chemical shift anisotropy (CSA). This CSA largely masks ionization dependent changes in chemical shift. Fortunately, an NMR technique called magic angle spinning (MAS) can be used to obtain the isotropic chemical shift from systems with a large chemical shift anisotropy such as multi lamellar vesicle (MLV) dispersions [30] and allows a more comprehensive understanding of the ionization behavior of PA in essentially flat bilayers [31].

The experimental procedure is as follows: individual samples are prepared by mixing lipids in organic solvent and subsequently drying the solutions down to a dry lipid film. This is usually accomplished by means of a stream of inert gas (often N_2) or by use of a rotary evaporator. These dry lipid films are then hydrated in buffer of specific pH and the samples are vortexed to disperse the lipid into the buffer as MLV's. The pH of these MLV dispersions is then measured and taken as the bulk pH of the sample. The lipid is then concentrated by means of centrifugation and the lipid pellet is transferred to the MAS NMR rotor after which the CS of PA is measured as a function of bulk pH of the sample. The resulting CS vs. pH titration curves can then be fitted by an equation derived from the Henderson–Hasselbalch equation to obtain the pK_a's (Eqs. (1) and (2)). Two schemes have been used. The first approach (using Eq. (1), [32]) results in the determination of both pK_a values and is valid if the CS is measured over a wide enough pH region ($1<pH<10$).

$$\delta = \frac{\delta_{AB} + \delta_{AA}*10^{pKa_1 - pH} + \delta_{BB}*10^{pH - pKa_2}}{1 + 10^{pKa_1 - pH} + 10^{pH - pKa_2}}. \quad (1)$$

Here δ is the measured chemical shift, and δ_{AA}, δ_{AB} and δ_{BB} are the chemical shifts of the fully protonated, singly protonated and fully dissociated phosphomonoester, respectively. Eq. (1) was successfully used to determine both pK_a values for low concentrations of PA in a PC bilayer [22]. However, this approach is not always practical and it is often more convenient to determine the second pK_a only. This is justified since this step in ionization (1 to 2 negative charges) is associated with the largest jump in CS values and most importantly the analysis does not significantly change the value obtained for pK_{a2} [22]. pK_{a2} is determined by fitting Eq. (2) to the data.

$$\delta = \frac{\delta_A*10^{pKa - pH} + \delta_B}{1 + 10^{pKa - pH}}, \quad (2)$$

where δ is the measured chemical shift and δ_A and δ_B are the chemical shifts of the singly dissociated and fully dissociated phosphomonoester, respectively. The degree of ionization of the phosphomonoester headgroup at any given pH can now be calculated using:

$$\%\ \text{ionization} = \frac{100}{1 + 10^{pKa_2 - pH}}. \quad (3)$$

4.2. The negative charge of PA is influenced by hydrogen bonds

We used the above protocol and found that despite identical phosphomonoester headgroups, PA and the related lipid lyso-phosphatidic acid (LPA, Fig. 1) have a non-identical ionization constant in the physiological pH range (Fig. 2; [22]). The titration curve for LPA in phosphatidylcholine (PC) bilayers is clearly shifted towards lower pH values, indicating that at a particular pH value, LPA will carry more negative charge than PA. The only difference between PA and LPA is the fact that LPA lacks an acyl-chain at the *sn*-2 position of the glycerol backbone, which is now occupied by a free hydroxyl

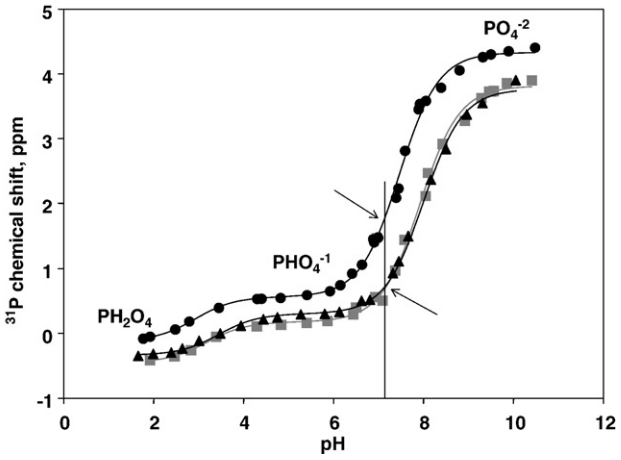

Fig. 2. ^{31}P MAS NMR titration curves for PA, LPA and dehydroxy-LPA. Circles, LPA; squares, PA; and triangles dehydroxy-LPA. The chemical shift is plotted as a function of pH, with 85% H_3PO_4 as a reference. Reproduced with permission from E.E. Kooijman et al., Biochemistry (2005) 44: 17007–17015.

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group (Fig. 1). The question that arose was whether or not this hydroxyl group was responsible for the difference in titration behavior between LPA and PA. In order to investigate this possibility, the titration behavior of a LPA compound lacking the *sn*-2 hydroxyl group (subsequently named, dehydroxy-LPA, Fig. 1) was determined and showed complete overlap with PA. These results indicated that the hydroxyl group of LPA is somehow able to lower the pK_{a2} of LPA compared to PA. Additional results for PA in a phosphatidylethanolamine (PE) bilayer indicated that PE lowered the pK_{a2} of PA (as well as LPA, [22]). These seemingly unrelated results led to the following model for the ionization properties of the phosphomonoester headgroup of PA:

> *Upon deprotonation of the first hydroxyl group the second proton in the phosphomonoester becomes more tightly bound because it is now shared between two hydroxyl oxygens of the phosphomonoester headgroup (see Figure 1). Any hydrogen bond formed with the phosphate headgroup will destabilize this second proton by competing for oxygen electrons, thereby facilitating its dissociation.*

This is indeed what is observed for PA, LPA, and dehydroxy-LPA (Fig. 2). In a PC bilayer, the pK_{a2} of LPA is lower than that of PA, indicating that at a particular pH value, there are more LPA molecules than PA molecules that carry two negative charges. We proposed that the free hydroxyl in LPA forms an intra-molecular hydrogen bond with the phosphomonoester headgroup, facilitating deprotonation. Such a hydrogen bond is observed in the LPA crystal structure [33], and is apparently preserved in the fully hydrated lipid membrane.

The observation that the pH titration curve of PA and dehydroxy-LPA overlapped excludes the possibility that the chain composition affected the ionization behavior of PA. In fact, dehydroxy-LPA, like PA, is expected to have a smaller molecular area due to the presence of only one acyl chain. A smaller molecular area results in an increase in charge density of the lipid and would subsequently be expected to give rise to an increase in pK_{a2} (i.e. decrease in charge at a specific pH). This is observed for neither LPA nor dehydroxy-LPA. Recent results on the pH titration behavior of two distinct acyl-chain species of PI(4,5)P_2 confirm this observation (Kooijman and Gericke, unpublished results). Thus while acyl-chain differences will result in small differences in lipid molecular area these differences are not expected to be have a significant effect on the ionization state of PA.

The ionization model was further supported by the results obtained using PE bilayers [22]. PE differs from PC in that PE carries a primary amine while PC has a quaternary amine in its headgroup. The primary amine should be able to form a hydrogen bond with PA and thereby facilitate deprotonation, and indeed, this is exactly what was observed. Interestingly, this hydrogen bond model, which can be thought of as an electrostatic-hydrogen bond switch (see below), also nicely explains the observation that LPA and PA have essentially the same pK_{a2} in a bilayer rich in PE. In such a bilayer, the large amount of hydrogen bond donors provided by the amine group of PE overrules the hydrogen bonding ability of the hydroxyl at the *sn*-2 position of LPA.

Note that the effect of PE on the ionization properties of PA implies that the negative charge of PA will vary depending on the intracellular location of PA as a function of the local PC:PE ratio in the membrane. This may have important physiological implications. For example, PA effectors may be recruited to specific intracellular compartments based on the surface charge density, which in case of PA, is regulated by the PC:PE ratio of the compartment. Additionally, PA formed in the cytoplasmic leaflet of the Golgi will carry more negative charge than PA present in the luminal leaflet due to the large difference in the PC:PE ratio (~8-fold lower in the cytoplasmic leaflet) and pH (~7.2 at the cytoplasmic leaflet and ~6.0 in the Golgi lumen). PA present in the Golgi luminal leaflet will have a larger negative curvature (smaller effective headgroup) than PA present in the cytoplasmic leaflet. Thus, if PA were formed in the cytoplasmic leaflet and is able to translocate to the lumen this might facilitate the fission of transport carriers formed at the Golgi. In short, the PC:PE ratio and its effect on the negative charge of PA may well regulate the function of PA in that specific intracellular location. The different functions and physiological roles of PA are further discussed in the additional reviews in this special BBA edition.

These results, while compelling, did not directly prove that hydrogen bonds influence the negative charge of PA. This issue was subsequently addressed when we followed up the initial ionization studies and investigated the effect of two amphiphiles, namely, dodecyltrimethylammonium (quaternary amine-no H-bonds) and dodecylamine (primary amine-H-bonds) [34]. These amphiphiles carry the same positive charge but differ in their ability to form hydrogen bonds, i.e. act as a proton donor. The results shown in Fig. 3A clearly indicate that the positive charge carried by dodecyltrimethylammonium increased the negative charge of PA (shift the CS to down field values) in accordance with Gouy–Chapman theory. However,

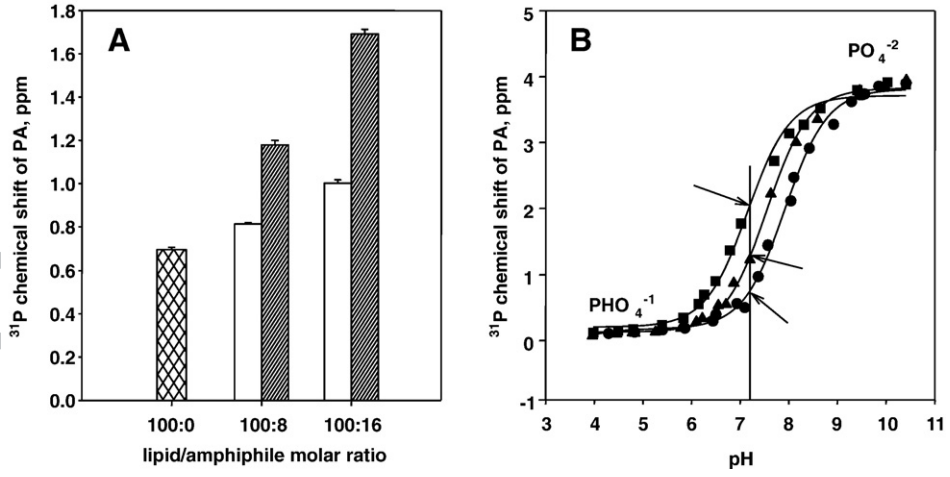

Fig. 3. Distinguishing between the effects of positive charge and hydrogen bond formation on the ionization properties of PA. (A) effects of the amphiphiles dodecyltrimethylammonium (white bars) and dodecylamine (dashed bars) on the chemical shift of PA at two lipid-to-amphiphile molar ratios; control without amphiphiles (checkered bar). (B) pH titration curves for PA in PC/PA (9:1, molar ratio) bilayers containing KALP23 (squares) at a lipid to KALP23 molar ratio of 25, DOTAP (triangles) at a lipid to DOTAP molar ratio of 6.25. The control (circles) is also shown. The arrows emphasize the differences in charge at pH 7.2. Reproduced with permission from E.E. Kooijman et al., JBC (2007) 282:11356–11364.

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dodecylamine, which in addition to the positive charge has the ability to form hydrogen bonds, increased the negative charge of PA by an additional ~60%.

These results were further substantiated by the use of more biologically relevant compounds, namely, the α-helical transmembrane peptide, KALP23 and the positively charged lipid, DOTAP. Titration curves in Fig. 3B show that at an identical positive charge density the effect of KALP23, flanked by lysine residues, is again ~60% larger than that of DOTAP, which carries the same charge as a lysine residue but is unable to form a hydrogen bond.

4.3. Working model of PA–protein interaction: the electrostatic-hydrogen bond switch model

The results described above thus provide compelling evidence supporting the idea that the negative charge of the phosphomonoester in PA is increased by the formation of intermolecular hydrogen bonds. They culminate in a model for PA–protein interaction that at the same time, captures the unique ionization properties of PA. The model was termed the electrostatic-hydrogen bond switch model as depicted in Fig. 4.

Basic residues in a PA binding domain are initially recruited to their target membrane by electrostatic attraction to anionic lipids. When the PA binding domain finds the anionic membrane and binds to it, the basic amino acid residues randomly sample their environment. There, they interact with hydrogen bond with negatively charged phosphodiesters (−1) which are present at much higher concentrations than PA. However, as soon as a basic residue comes into close proximity (<3.5 Å) to PA, a hydrogen bond is formed leading to a further deprotonation (to −2) of the headgroup of PA. This is depicted in the right of Fig. 4. The subsequent increase in negative charge of the phosphomonoester headgroup of PA coupled with hydrogen bonding, now locks the side chains of the basic residues on the headgroup of PA resulting in tight docking of the membrane interacting protein on a di-anionic PA molecule. This model clearly describes how the phosphomonoester headgroup sets PA apart from all other anionic glycerophospholipids.

5. Literature supporting the electrostatic-hydrogen bond switch model

Below we review recent literature on protein–lipid interaction that supports the electrostatic-hydrogen bond switch model. These data not only appear to validate the model for PA, but further extends its general validity to all phosphomonoesters.

5.1. The case of the sphingolipid ceramide-1-phosphate

If the electrostatic-hydrogen bond switch model is a general model for the ionization properties of phosphomonoesters, additional lipids and compounds carrying a phosphomonoester should show an identical ionization behavior. A prime question is then: is this indeed the case?

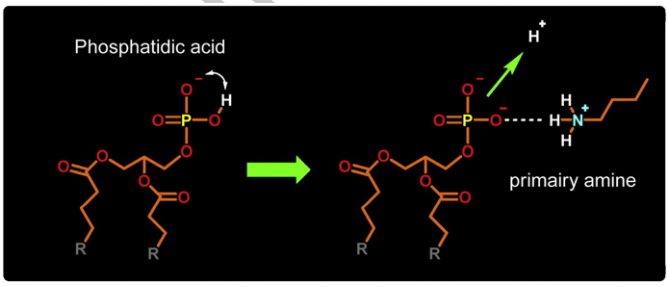

Fig. 4. The electrostatic-hydrogen bond switch model.

The short answer is, yes; recent data on the related lipid ceramide-1-phosphate (Cer-1-P) which is the sphingolipid counter part of the glycerol based lipid PA confirms the electrostatic-hydrogen bond switch model. The pK_{a2} of Cer-1-P was determined and found to be very close to LPA, and quite different from PA [35]. Similarly, PE decreased the pK_{a2} of Cer-1-P in a fashion identical to the effects of PE on (L)PA [35]. These results can be understood considering the structure of Cer-1-P (see Fig. 1), which, like LPA, carries a hydroxyl group in its backbone. This hydroxyl group will hydrogen bond to the phosphomonoester, decreasing pK_{a2} [35]. Recent data obtained on other lipids containing phosphomonoesters, including the polyphosphoinositides, confirm the general validity of the electrostatic-hydrogen bond switch model for the ionization properties and protein–lipid interaction of lipid phosphomonoesters (E.E. Kooijman and A. Gericke, unpublished observations).

5.2. Computational support for the electrostatic-hydrogen bond switch model

Additional support for the electrostatic-hydrogen bond switch model comes from recent experimental and computational work by Tigyi and Parrill on LPA and sphingosine-1-phosphate (S1P) receptors [36]. Lysophosphatidic acid and S1P are important signaling molecules that bind to and activate specific G protein coupled receptors (GPCRs) in the plasma membrane and nuclear membrane of cells. Interestingly, the binding of LPA and S1P to their respective GPCR depends critically on the phosphomonoester headgroup of these molecules [37,38], and the binding region of the LPA receptors contains two conserved basic residues, a lysine and arginine [38].

Our results described above for PA predict that LPA and S1P should bind their receptor in di-anionic form. Indeed, this is exactly what computational work by Tigyi and Parrill has shown for S1P binding to the $S1P_1$ receptor [36]. Importantly, the computational analysis clearly shows that an intra-molecular hydrogen bond between the phosphomonoester headgroup of S1P and the free hydroxyl group in the backbone (identical to the situation for Cer-1-P described above) stabilizes the fully deprotonated form of S1P. The fact that conformations of S1P that lack the intra-molecular hydrogen bond have a higher energy, indicates that intramolecular hydrogen bond formation in S1P is energetically favorable, and the same is expected for LPA and Cer-1-P. Similar types of intra-molecular hydrogen bond formation have been observed or suggested for the myo-inositol phosphates [39,40].

5.3. Interaction of basic protein domains in transmembrane proteins with PA

Compelling support for the model comes from the lipid interaction of clusters of basic residues in transmembrane proteins. An important feature of many transmembrane proteins is that their transmembrane segments are flanked on the cytosolic side of the membrane by positively charged residues. These residues are thought to stabilize the transmembrane orientation of the protein and/or regulate its function [17,41–44]. The model predicts that such regions, given their favorable localization in the lipid headgroup region of the membrane, should have a preferential interaction with PA. Recent evidence suggests that this is indeed the case for several transmembrane ion channels [45–47].

MscL is the mechanosensitive channel of large conductance, and carries a cluster of three basic amino acids (R98, K99, and K100) on its cytosolic face. Tryptophan fluorescence quenching studies showed that this basic cluster has a specific, high affinity, interaction with PA [46]. Similarly, computational studies of the bacterial potassium channel KcsA show that PA has a special interaction with basic residues in the interface between the monomers of the tetrameric KcsA channel [45]. This interaction between PA and KcsA was

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confirmed in TFE unfolding studies, and special electrostatic and hydrogen bond interactions probably explain the stabilizing effect of PA on the KcsA tetramer [47].

5.4. C2 domain of protein kinase C interacts with PA

Recent work by Gomez-Fernandez and co-workers on the peripheral membrane protein, protein kinase C (PKC), in particular the α and ε isoforms [48,49], also provides tantalizing evidence for the model. PKC proteins are targeted to the membrane via a C2 domain, a membrane binding module with a lipid specificity that depends on the particular PKC isoform [50,51]. In the case of the α isoform of PKC, x-ray crystallography shows a PA molecule located at a polybasic region of the C2 domain flanking the lipid bilayer [48], consistent with the electrostatic-hydrogen bond switch model. Previously, Gomez-Fernandez and co-workers showed that PKCε had specificity for PA [52,53], and recently used MAS NMR to evaluate this interaction [49]. They suggest that upon binding PKCε the charge of PA is indeed increased to −2, in agreement with the electrostatic-hydrogen bond switch model.

5.5. Small molecule interactions with PA

Docking of basic amino acid residues on the headgroup of PA likely provides an important mode of PA–protein interaction as described by the electrostatic-hydrogen bond switch model. However, the model is clearly not limited to amino acid–lipid and lipid–lipid interactions. Additional moieties that provide a positive charge and a hydrogen bond donor should suffice. Recent work from the group of Kinnunen in Finland provides a tantalizing glimpse at the possibilities [54]. In their studies of siramesine, a potent σ-2 receptor agonist and cancer cell growth inhibitor [55,56], a highly specific high affinity interaction with PA was observed. MD simulations suggest a strong electrostatic hydrogen bond interaction between the phosphomonoester of PA and a positively charge amine of siramesine. An additional feature of siramesine–PA interaction was revealed in lipid monolayer studies in which the degree of insertion of siramesine as a function of surface pressure (a measure of lipid packing) was studied for different lipid mixtures. Siramesine insertion was dramatically increased by inclusion of PA in the PC monolayer, while inclusion of PS had an inhibitory effect. Overall, the work by Kinnunen may provide a new paradigm for the development of small anticancer drugs which act by specific sequestering of lipid second messengers. The electrostatic-hydrogen bond switch model for the ionization properties of a phosphomonoester headgroup may provide important new leads in drug target discovery as well as in drug development.

6. Biological implications and predictions of the electrostatic-hydrogen bond switch model

The model described above not only describes the electrostatics and interactions of PA. We already saw that the negative charge of Cer-1-P and PA is modulated in an identical fashion, if one takes the differences in chemical structure between Cer-1-P and PA into account. In fact the electrostatic-hydrogen bond model describes the ionization properties and modes of interaction for any phosphomonoester moiety. Phosphomonoester groups are ubiquitous in nature, occurring not only in lipids but also in many proteins where (de) phosphorylation often switches enzymes between active and inactive states. Recognition and binding of most, if not all phosphomonoester moieties by their binding partners are probably governed by the charge modulation and hydrogen bond formation as described by the electrostatic-hydrogen bond switch model.

In the case of proteins binding to a phosphomonoester moiety, the hydrogen bond donor is often a lysine or arginine residue. But this is not an absolute requirement as exemplified by the PA binding domain of protein phosphatase 1 (PP1 [57]). Amino acid mutations in the binding site of PP1 showed that a serine residue (which has a –OH as side group) in close proximity to a basic region was critical for the binding of PA. Therefore, while lysine and arginine clusters are usually the hydrogen bond donors for binding to PA, there is no absolute requirement for a positive charge on the hydrogen donor itself. A more general combination of hydrogen bond donor and positive charge nearby is sufficient to fulfill the electrostatic requirements of PA binding.

6.1. PA is the preferred anionic lipid for the interfacial insertion of basic protein domains

The docking of basic protein domains on PA may be followed by insertion of hydrophobic protein domains into the hydrophobic interior of the lipid bilayer. One example of such a favorable hydrophobic interaction has been described in vitro for the GTPase dynamin. Dynamin, which binds to negatively charged membranes, shows considerably more insertion in mixed-lipid monolayers containing PA as compared to other negatively charged phospholipids (the molecular area of insertion is highest in the presence of PA; see [58]).

How can we understand these hydrophobic interactions? On top of its high charge and capacity to form hydrogen bonds, (unsaturated) PA also has a special "effective molecular shape" [31,59]. The idea of effective molecular shape is nicely reviewed by Mouritsen [60]. With the possible exception of Cer-1-P which occurs in even smaller (than PA) amounts in cells [35], PA is the only anionic phospholipid with a pronounced cone shape (negative curvature) under physiological conditions [44]. Cone shaped lipids facilitate protein penetration into the membrane by reducing lipid head group packing, forming favorable insertion sites in the headgroup region of the lipid bilayer [61].

The effect of cone shaped lipids has been investigated *in vitro* for the well known PA binding domain of Raf-1 kinase (RPA, [62–64]) and for the protein Constitutive response 1 (CTR1, [65]). Indeed, the cone shaped lipid PE was found to strongly increase PA binding of the PA binding domain of Raf-1 [34]. This binding depended critically on the presence of PA. The mere presence of hydrophobic insertion sites (50% PE) did not lead to efficient binding of the RPA domain, and RPA bound at least 2.5 fold more upon inclusion of 20 mol% PA when compared to 20 mol% PS [34]. Lipid-binding experiments with the PA-binding protein CTR1 support this notion [65]. The plant protein CTR1, is a key regulator of ethylene signaling [66–68] and a close homologue of the mammalian PA-binding protein Raf-1 kinase [62–64], and specifically binds vesicles containing PA *in vitro* [65]. Moreover, replacing half of the PC background with PE significantly increased binding of CTR1 to the PA-containing vesicles (C. Testerink, unpublished data).

The effective molecular shape of PA likely explains the difference in binding and insertion of the potential anti cancer drug siramesine to lipid monolayers (and bilayers). Compared to PS, PA has an effective molecular shape that facilitates the interfacial insertion of siramesine. Thus, docking of siramesine on the phosphomonoester of PA coupled to the interfacial insertion of hydrophobic parts of the molecule is most likely responsible for the specificity and high affinity of lipid interaction.

Finally, the observation that PA may act as a docking site for membrane interacting peptides [34] very close to the hydrophobic interior of the lipid bilayer, together with the cone shape of PA, turns PA into a very effective insertion site for positively charged membrane-active proteins. A commonly overlooked possibility for negatively charged membranes may play an important role in this regard as well. The protons absorbed to the negatively charged membrane (see above) may protonate acidic residues, such as aspartic and glutamic acid, and facilitate their insertion in the hydrophobic interior of the membrane. A corollary may be that the binding of a PA

Please cite this article as: E.E. Kooijman, K.N.J. Burger, Biophysics and function of phosphatidic acid: A molecular perspective, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.04.001

binding domain to PA liberates a proton from the headgroup of PA and this proton may act to protonate an acidic residue and facilitate its insertion.

Experimental and computational data found in the literature to date suggest that the electrostatic-hydrogen bond switch in the phosphate headgroup of PA coupled to the location of the phosphate headgroup very close to the hydrophobic interior of the lipid bilayer [34], sets PA apart from all the other anionic membrane lipids.

6.2. A specific PA binding domain structure?

The data and literature thus raise the question what a PA binding domain would look like? As stated in the introduction, the literature thus far (reviewed in [6,7]), does not show a general amino acid sequence motif responsible for PA binding. This is contrary to proteins that bind lipids like PI(4,5)P_2, where specific domains have evolved [16]. These relatively large cage-like domains get easy access to the inositol(poly)phosphate headgroup because it is located right at the water–headgroup interface. The case for PA is dramatically different because the headgroup is located very close to the headgroup–acyl chain interface and large protein domains do not easily fit around PA. This nicely explains why PA-binding proteins appear to make use of the unique electrostatic properties and effective molecular shape of PA. Whether or not there are any structural similarities between the different PA binding domains remains to be elucidated. It is interesting to note a possible corollary of the binding of PA by PA-binding domains. While most PIP_2 binding proteins have special cage-like binding domains [16], several important exceptions have recently emerged. These proteins, like PTEN [69], or NAP-22 [70,71] have domains that in many ways resemble the PA binding domains, in that no clear structure or large protein domain is present. These peptides are likely to bind to PIP_2 utilizing the electrostatic-hydrogen bond switch provided by the phosphomonoester(s) in the headgroup of PIP_2. Many fascinating discoveries concerning the binding of proteins to lipid phosphomonoesters are likely to emerge in the years to come.

Acknowledgments

The authors would like to thank Ben de Kruijff for the many insightful discussions that led to the work described in this review. EEK acknowledges financial support from Kent State University. The authors apologize to those authors whose original work is not cited here due to length considerations. Most original works can be found in the reviews mentioned.

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Please cite this article as: E.E. Kooijman, K.N.J. Burger, Biophysics and function of phosphatidic acid: A molecular perspective, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.04.001

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